

## Ontogeny of Eyeblink Conditioned Response Timing in Rats

John H. Freeman Jr., Daniel A. Nicholson, Adam S. Muckler, Christine A. Rabinak, and Norma T. DiPietro  
University of Iowa

Eyeblink conditioned response (CR) timing was assessed in adult and infant rats. In Experiment 1, adult rats were trained with a 150-ms tone conditioned stimulus (CS) paired with a periorbital shock unconditioned stimulus (US; presented at 200- or 500-ms interstimulus intervals [ISIs]). The rats acquired CRs with 2 distinct peaks that occurred just before the US onset times. Experiments 2 and 3 examined developmental changes in CR timing in pups trained on Postnatal Days 24–26 or 32–34. Experiment 3 used a delay conditioning procedure in which the tone CS continued throughout the ISIs. Pups of both ages exhibited robust conditioning. However, there were age-related increases in the percentage of double-peaked CRs and in CR timing precision. Ontogenetic changes in eyeblink CR timing may be related to developmental changes in cerebellar cortical or hippocampal function.

The conditioned eyeblink can be thought of as a temporally adaptive response to an aversive stimulus. The eyeblink conditioned response (CR) is initiated before the onset of the unconditioned stimulus (US), and the peak amplitude of the CR occurs at or just before the onset time of the US (Gormezano, 1966; Gormezano, Kehoe, & Marshall, 1983). Several conditioning procedures have been used to examine the precision and limits of CR timing. The most straightforward method for examining the temporal specificity of the CR is to vary the interstimulus interval between groups of subjects. The topography of the eyeblink CR varies systematically with interstimulus interval, and the peak of the response occurs at the onset of the US for each of the intervals (Gormezano et al., 1983; Schneiderman & Gormezano, 1964; Smith, 1968; Smith, Coleman, & Gormezano, 1969).

Response timing has also been examined in paradigms that use multiple interstimulus intervals (ISIs) in the same subjects. Millenson, Kehoe, and Gormezano (1977) used a tone CS that varied in duration, producing 200- and 700-ms ISIs. Different groups received different proportions of trials with the 200- or 700-ms ISI. Rabbits in the 50/50 condition developed double-peaked CRs. That is, when the longer CS was presented, the well-trained rabbits produced CRs with peak amplitudes at 200 and 700 ms following CS onset. Mauk and Ruiz (1992) used a within-subjects design to present two distinct conditioned stimuli (CSs) that differed in duration, yielding two ISIs (150 and 750 ms). Rabbits developed CRs that were timed appropriately to the onset of the US that was paired with each CS. A third paradigm that has been used to assess CR timing used a single CS that was paired with a US, which was presented at two different ISIs (300 and 700 ms, Choi, 1999; Moore & Choi, 1997). This “temporal uncer-

tainty” paradigm yields CRs that have two peaks occurring at the onset times of the US.

The results of experiments using the timing paradigms described above have stimulated experimental and theoretical analyses of the neural mechanisms of eyeblink CR timing (Bullock, Fiala, & Grossberg, 1994; Fiala, Grossberg, & Bullock, 1996; Garcia & Mauk, 1998; Mauk & Donegan, 1997; Medina, Garcia, Nores, Taylor, & Mauk, 2000; Medina & Mauk, 2000; Moore & Choi, 1997; Moore, Choi, & Brunzell, 1998; Perrett, Ruiz, & Mauk, 1993). A generally held view is that the cerebellar cortex mediates the precise timing of the CR and directs the activity of the cerebellar nuclei. The cerebellar nuclei provide the output of the cerebellum to the red nucleus, which projects to the motor neurons that control the eyeblink response. Several lines of evidence support the view that the cerebellar cortex plays a necessary role in response timing. Lesions of the cerebellar cortex that spare the underlying deep nuclei disrupt CR timing, resulting in short-latency responses that do not peak at the onset time of the US (McCormick & Thompson, 1984; Perrett et al., 1993). Moreover, pharmacological disconnection of the cortex from the deep nuclei with picrotoxin reversibly impairs CR timing (Garcia & Mauk). Unit recordings of Purkinje cell activity and neuronal activity in the interpositus nucleus during the temporal uncertainty paradigm indicate very tight coupling between cerebellar neuronal activity and CR topography (Choi, 1999; Moore & Choi). The unit recordings in the cerebellar cortex indicate that some Purkinje cells disinhibit the deep nuclei just before each of the two peaks of the CR (Choi; Moore & Choi). The lesion, inactivation, and unit recording data have been incorporated into computational models of eyeblink conditioning that specifically address the role of the cerebellar cortex in CR timing (Bullock et al.; Fiala et al.; Mauk & Donegan; Medina et al.; Medina & Mauk; Moore & Choi; Moore et al.). These models suggest that CR timing is mediated by learning-specific synaptic plasticity between parallel fibers and Purkinje cells and the interactions of Purkinje cells with neurons in the interpositus nucleus. The empirical and theoretical analyses of eyeblink CR timing provide compelling evidence implicating cerebellar cortical function in response timing.

The hippocampus has also been implicated in CR timing. Lesions of the hippocampus result in short-latency CRs when cou-

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John H. Freeman Jr., Daniel A. Nicholson, Adam S. Muckler, Christine A. Rabinak, and Norma T. DiPietro, Department of Psychology, University of Iowa.

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Correspondence concerning this article should be addressed to John H. Freeman Jr., Department of Psychology, University of Iowa, E11 Seashore Hall, Iowa City, Iowa 52242. E-mail: john-freeman@uiowa.edu

pled with certain trace training procedures (James, Hardiman, & Yeo, 1987; Moyer, Deyo, & Disterhoft, 1990; Port, Romano, Steinmetz, Mikhail, & Patterson, 1986; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). However, several sources of evidence indicate that the hippocampus plays a transient role in trace conditioning. Posttraining lesions of the hippocampus disrupt retention of CRs when given shortly after trace conditioning, but not after the CR has been consolidated (Kim, Clark, & Thompson, 1995). Neuronal activity in the hippocampus shows learning-specific alterations primarily at the beginning of trace conditioning (as CRs first emerge), which decrease with continued training (McEchron & Disterhoft, 1997). Learning-specific increases in hippocampal pyramidal cell membrane excitability are also transient, decreasing over time or extended training (Moyer, Thompson, & Disterhoft, 1996). The cited studies indicate that the hippocampus plays a transient role in trace conditioning and CR timing under certain training conditions. If the hippocampus plays a role in CR timing after consolidation, it may influence timing by providing relevant temporal information to the cerebellum.

Developmental studies of eyeblink conditioning in infant rats have indicated that basic delay conditioning emerges between Postnatal Days 17 and 24 (Stanton, Freeman, & Skelton, 1992). Neurobiological studies of the ontogeny of delay conditioning indicate that there are substantial developmental changes in synaptic connections between neurons in the brainstem and cerebellar circuitry that is necessary and sufficient for conditioning (Freeman & Nicholson, 2000, 2001; Nicholson & Freeman, 2000). However, there may be more protracted developmental changes in cerebellar cortical or hippocampal function that extend beyond Postnatal Day 24. For instance, Purkinje cell dendrites and synaptic contacts continue to develop past Postnatal Day 30 in rats, indicating that synapses continue developing after the age at which rats develop robust delay conditioning (Anderson & Flumerfelt, 1985; Berry & Bradley, 1976). Hippocampal neurons also exhibit extensive morphological and physiological maturation postnatally (Cotman, Taylor, & Lynch, 1973; Dumas & Foster, 1995; Harris & Teyler, 1984; Pokorny & Yamamoto, 1981a, 1981b). Developmental changes in response timing during training with temporal uncertainty paradigms would suggest that there are protracted developmental changes in cerebellar cortical or hippocampal function.

The current study was designed to examine timing of eyeblink CRs in adult rats and to assess developmental changes in CR timing in infant rats. Experiment 1 examined CR timing in adult rats that were given temporal uncertainty training with an auditory CS (150 ms) that was paired with a periorbital shock US (50 ms) at one of two ISIs (200 or 500 ms). This experiment was designed to extend the findings of temporal uncertainty training in adult rabbits to rodents (Moore & Choi, 1997). Experiment 1 also provided a comparison of the precision of response timing using eyelid movement (Moore & Choi, 1997) and eyelid electromyographic (EMG) activity. Experiment 2 was designed to examine developmental changes in the precision of CR timing. Rats were given the same temporal uncertainty training as in Experiment 1 on Postnatal Days 24–26 or 32–34. Experiment 3 used a delay conditioning paradigm with two different CS durations (i.e., two different ISIs; Millenson et al., 1977) to further examine developmental changes in CR timing.

## Experiment 1

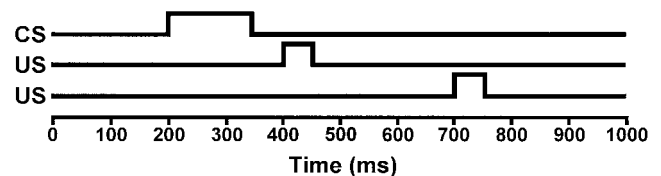
The precision of eyeblink CR timing has been examined in rabbits, using restraint and precise measurement of eyelid or nictitating membrane movement (Mauk & Ruiz, 1992; Millenson et al., 1977; Moore & Choi, 1997). Timing of CRs has not been examined in detail in freely moving rodents, nor has timing of eyelid EMG activity. It is possible that only restrained subjects would demonstrate precisely timed CRs, perhaps simply because of lower CR variability in comparison to unrestrained subjects. Eyelid EMG activity might also be a measurement that is too variable relative to eyelid (or nictitating membrane) movement to allow detection of precise timing. On the other hand, the more rapid fluctuations in EMG activity relative to eyelid movement might more clearly reveal the precision of CR timing. The precision of eyelid EMG CR timing and the utility of the rodent paradigm for studies of CR timing in general were examined in Experiment 1.

The utility of the rodent paradigm for evaluating CR timing also has implications for evaluation of the relationship between gene function and learning. Most of the mammalian work in gene manipulation has occurred in rodents. Moreover, many of the genetic manipulations that influence eyeblink conditioning involve alterations of cerebellar cortical function (Aiba et al., 1994; Chen, Bao, Lockard, Kim, & Thompson, 1996; De Zeeuw et al., 1998; Shibuki et al., 1996; Wemmie et al., 2002). The precise role of genes that are expressed in the cerebellar cortex in learning may therefore be elucidated by careful examination of CR timing.

In Experiment 1, adult rats were trained with a slightly modified version of a temporal uncertainty paradigm previously used with rabbits (Moore & Choi, 1997). The rats were presented a 150-ms tone CS that was paired with a 50-ms periorbital shock US at ISIs of 200 or 500 ms (see Figure 1). Daily training sessions consisted of 100 trials, with nonreinforced presentations of the CS on every 10th trial. The CS-alone test trials were used to assess longer latency components of the CR that would be contaminated by the unconditioned response. The two types of trials were presented in an irregular sequence. If the rat eyeblink CR is timed similarly to the rabbit CR (Choi, 1999; Moore & Choi, 1997), there should be two peaks of EMG activity that correspond to the two ISIs (i.e., 200 and 500 ms after CS onset).

## Method

*Subjects.* Subjects were 7 male Long–Evans rats (250–350 g). The rats were housed in the animal colony in Spence Laboratories at the University



*Figure 1.* Diagram of the trace conditioning procedure used in Experiments 1 and 2. The time course of the conditioned stimulus (CS) and the two different onset times of the unconditioned stimulus (US) are depicted by the solid lines. The CS was presented on every trial, and the US was presented at one of the two onset times on each trial. Note the time gap, or trace interval, between the offset of the CS and the onsets of the US.

of Iowa. All rats were maintained on a 12-hr light–dark cycle (light onset at 7 a.m.) and given ad-lib access to food and water.

**Surgery.** One week prior to training, rats were removed from their home cage and anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and atropine sulfate (0.45 mg/kg). At the onset of anesthesia, the rats were fitted with differential EMG electrodes that were implanted in the left upper eyelid muscle (orbicularis oculi). A ground electrode was attached to a stainless steel skull screw. The EMG electrode leads terminated in gold pins in a plastic connector, which was secured to the skull with dental acrylic. A bipolar stimulating electrode (Plastics One, Roanoke, VA) for delivering the shock US was implanted subdermally, immediately caudal to the left eye. The bipolar electrode terminated in a plastic connector that was secured to the skull by dental acrylic. The surgical site was closed with sutures on both sides of the electrode connectors. The rats were given 1 week to recover from surgery before training began.

**Apparatus.** The conditioning apparatus consisted of four small-animal sound-attenuating chambers (BRS/LVE, Laurel, MD). Each sound-attenuating chamber contained a small-animal operant chamber (BRS/LVE) in which the rats were held during conditioning. One wall of the operant chambers was fitted with two speakers that independently produce tones of up to 120 db (SPL), with a frequency range of approximately 1000–9000 Hz. The back wall of the sound-attenuating chambers was equipped with a small light. The connectors for the EMG electrodes and bipolar stimulating electrode were connected to lightweight cables and a commutator that allowed the rats to move freely during training. The electrode leads from the rat’s head stage were connected to peripheral equipment and a desktop computer. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity. Eyelid EMG activity was recorded differentially, bandpass filtered (500–5000 Hz), amplified, and integrated (2.5 ms bins for 1.0 s) by equipment that was similar to that used in previous studies (e.g., Freeman & Nicholson, 2000; Nicholson & Freeman, 2000, 2001; Stanton et al., 1992).

**Conditioning procedure.** The experimental design is illustrated in Figure 1. The rats were given 100-trial training sessions that included a 150-ms tone CS (2.0 kHz, 85 dB [SPL]) that was paired with a periorbital shock US (50 ms, 2.5 mA). The shock was delivered either 200 or 500 ms after the onset of the CS in an irregular sequence. This procedure differs slightly from that used by Moore and Choi (1997) in that there was a stimulus-free period between the tone offset and shock onset for both ISIs. The CS was presented alone on test trials every 10th trial. The rats were trained until they produced double-peaked CRs on at least 50% of the test trials for two consecutive training sessions. This criterion was based on the findings of a previous study (Millenson et al., 1977).

CRs were defined as EMG activity that exceeded a threshold of 0.4 amplified units above the baseline activity following onset of the CS. Double-peaked CRs were defined as CRs that had an initial increase of at least 1.0 EMG units, followed sequentially by a decrease of more than 0.5 units and a second increase of at least 1.0 units. This criterion was similar to that used for double-peaked conditioned nictitating membrane responses (Millenson et al., 1977).

**Results**

The rats acquired CRs rapidly during training and reached the criterion of 50% double-peaked CRs on two consecutive sessions in a mean of 6.4 training sessions (range = 4–8). On test trials during the last training session, the peaks of the eyelid EMG activity were precisely timed to the 200- and 500-ms onset times of the US (see Figure 2), with peak latencies of 175.3 (range = 154.3–212.9) and 434.2 (range = 388.6–491.7) ms, respectively. Thus, the mean peak latencies of the EMG activity preceded the onset times of the US by 24.7 and 65.8 ms. The amplitudes of the two peaks (3.14 and 2.38 units, respectively) differed significantly,

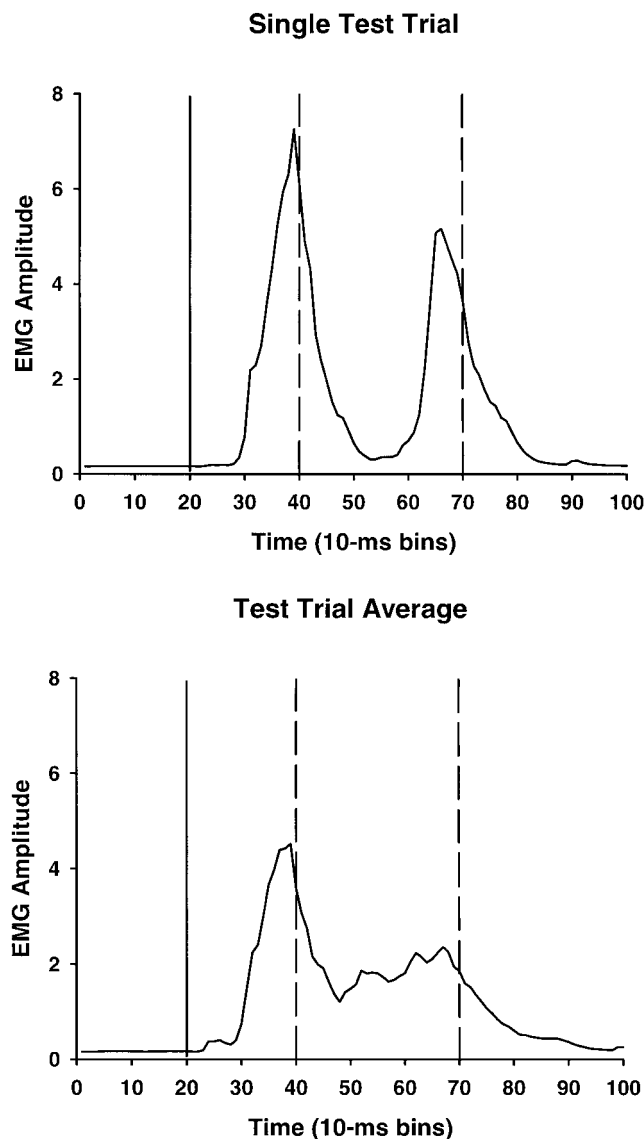


Figure 2. Electromyographic (EMG) activity from a rat on a single test trial (top) and averaged across test trials with double-peaked conditioned responses (bottom). The solid vertical line indicates the onset time of the conditioned stimulus. The two dashed vertical lines indicate the onset times of the unconditioned stimulus (Experiment 1).

$t(6) = 5.74, p < .05$ , with the first peak consistently larger than the second.

The CRs that were not classified as double-peaked were those with a single peak occurring primarily just prior to the first US onset time. The mean peak latency of the single-peaked CRs was 193.3 ms (range = 120.0–350.0), which was slightly delayed relative to the first peak of the double-peaked CRs.

**Discussion**

The results of Experiment 1 indicate that adult rats readily acquire precisely timed double-peaked CRs during temporal uncertainty training. The results of this experiment are generally

consistent with the results of temporal uncertainty experiments in rabbits (Moore & Choi, 1997). However, the peaks of the EMG responses preceded the onset times of the US, whereas the peak nictitating membrane or eyelid movement occurs at or just after the onset times of the US. This difference can be explained simply by noting that EMG activity precedes eyelid or nictitating membrane movement by 10–20 ms (Berthier, 1992; Pellegrini, Horn, & Evinger, 1995; Trigo, Gruart, & Delgado-Garcia, 1999). The greater amplitude of the first peak relative to the second peak of the eyelid response differs from the pattern seen in the rabbit paradigm and in simulations of the time-derivative model (Moore & Choi, 1997). According to this model, the second peak should be larger, and the reason for the amplitude difference is that the offset of the CS is an additional cue that contributes to the amplitude of the second peak, but not the first. In the present experiment, CS offset may contribute to both peaks because there was a time gap between the offset of the CS and the onset of the US at both intervals.

### Experiment 2

The eyeblink CR emerges ontogenetically between Postnatal Days 17 and 24 (Stanton et al., 1992). By Postnatal Day 24, the acquisition and asymptotic performance of the eyeblink CR is virtually indistinguishable from the performance of adult rats trained with similar procedures. In both adult and 24-day-old rats, the eyeblink CR peaks prior to the onset of the US. However, the precision and limits of CR timing have not been examined in infant rats.

The primary basis for examining developmental changes in CR timing is that the cerebellar cortex and hippocampus continue to develop after Postnatal Day 24 in rats (Altman, 1982). In fact, the neurons that are thought to be most intimately associated with CR timing in the cerebellar cortex, Purkinje cells, exhibit continued maturation past Postnatal Day 30 (Berry & Bradley, 1976). The late stages of Purkinje cell and hippocampal pyramidal cell maturation are characterized by an increase in dendritic branching, which is likely accompanied by an increase in synapses. Because synaptic plasticity in Purkinje cells and hippocampal pyramidal cells is thought to be a contributing mechanism to CR timing, it is possible that CR timing continues to develop between Postnatal Days 24 and 30.

Experiment 2 examined developmental changes in eyeblink CR timing in the same training procedure as in Experiment 1. The rats started training on either Postnatal Day 24 or Postnatal Day 32 and continued for 3 days. Each training day included three 100-trial sessions.

### Method

**Subjects.** Subjects were 15 Long-Evans rat pups trained on either Postnatal Days 24–26 ( $n = 8$ ) or 32–34 ( $n = 7$ ). One of the rats in the older age group did not complete the experiment. The rats were housed in the animal colony in Spence Laboratories at the University of Iowa. All rats were maintained on a 12-hr light–dark cycle (light onset at 7 a.m.) and given ad-lib access to food and water.

**Surgery, apparatus, and conditioning procedure.** The surgical procedures, apparatus, and conditioning procedure were identical to those used in Experiment 1, except that the infant rats were given three training sessions per day. The training sessions were separated by 4 hr. Repeated

daily training is necessary in infant rats because they exhibit very rapid neural and physical maturation during the 1st postnatal month. In fact, the rats typically outgrow and lose the head stage after 4 or 5 days. Moreover, the use of multiple training sessions per day in infant rats is a standard procedure that has been used in all of the reported studies of eyeblink conditioning in infant rats (e.g., Freeman, Spencer, Skelton, & Stanton, 1993; Freeman, Barone, & Stanton, 1995; Freeman, Carter, & Stanton, 1995; Freeman & Nicholson, 2000; Ivkovich, Paczkowski, & Stanton, 2000; Ivkovich & Stanton, 2001; Nicholson & Freeman, 2000, 2001; Stanton et al., 1992; Stanton, Fox, & Carter, 1998).

### Results

The two groups of rats acquired eyeblink CRs at the same rate in Experiment 2 (see Figure 3). Both groups rapidly acquired CRs and reached asymptotic levels of performance on Session 6. However, the younger rats exhibited fewer double-peaked CRs across Sessions 5–9 relative to the older rats (see Figure 4), which indicates that CR production and specific CR timing may develop at different rates. Examination of the latencies of the response peaks for trials with double-peaked CRs on the last training session (see Figure 5) revealed that the younger pups generated double-peaked CRs with a first peak that followed the first onset time of the US ( $M = 217.3$  ms, 17.3 ms after US onset) and a second peak that preceded the second onset time of the US ( $M = 477.9$  ms, 22.1 ms before US onset). The older rats produced CRs with peaks that both preceded the onset times of the US ( $M_s = 188.4$  and 455.8 ms, 11.6 and 44.2 ms before first and second US onset times, respectively), which is similar to the pattern of latencies in adults.

The percentage of CRs, the percentage of double-peaked CRs, and the peak latencies of the double-peaked CRs were examined between age groups by analysis of variance. There were no significant effects for the percentage of CRs between age groups,  $F(1, 12) = .09$ ,  $p = .78$ . The analysis of the percentage of double-peaked CRs revealed a significant interaction between age and sessions,  $F(8, 96) = 2.46$ ,  $p < .02$ . Post hoc tests (Tukey's honestly significant difference) revealed that the older rats exhibited significantly more double-peaked CRs than the younger rats

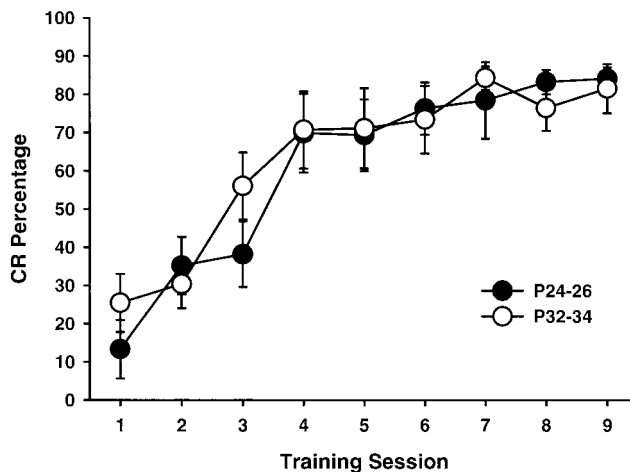


Figure 3. Mean ( $\pm$ SEM) percentage of conditioned responses (CRs) produced by rats trained on Postnatal Days (P) 24–26 or 32–34 as a function of 100-trial training sessions. The rats were given three training sessions per day (Experiment 2).

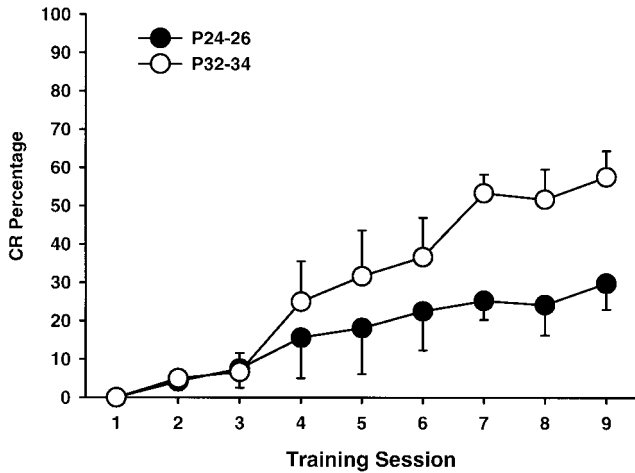


Figure 4. Mean ( $\pm$ SEM) percentage of double-peaked conditioned responses (CRs) produced by rats trained on Postnatal Days (P) 24–26 or 32–34 as a function of 100-trial training sessions. The rats were given three training sessions per day (Experiment 2).

during Sessions 7–9 (all comparisons,  $p < .05$ ). There was a significant effect of age for the analysis of peak latency for double-peaked CRs for the first peak,  $F(1, 12) = 5.19, p < .05$ . This effect was due to a longer latency first peak in the younger rats. The latency of the second peak did not differ between groups.

The responses of infant rats that were not classified as double-peaked CRs were responses with a single peak that occurred shortly after the first US onset time. The mean onset latencies for the two age groups were 234.8 ms in the younger rats and 256.4 ms in the older rats. The mean peak latencies for the single-peaked responses occurred later than the latency of the first peak of the double-peaked CRs in both age groups.

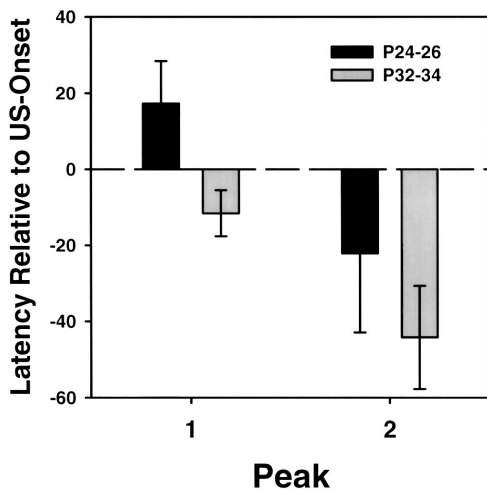


Figure 5. Mean ( $\pm$ SEM) latency of the first (left) and second (right) peak of double-peaked conditioned responses relative to the onset times of the unconditioned stimulus (US) in rats trained on Postnatal Days (P) 24–26 or 32–34 (Experiment 2).

Discussion

The results of Experiment 2 indicate that although rats rapidly acquire eyeblink CRs in a short-delay paradigm by Postnatal Day 24, the precision of CR timing continues to develop through Postnatal Days 32–34. The most significant developmental change in CR timing was an age-related increase in the percentage of double-peaked CRs (see Figure 4). The rats trained on Postnatal Days 24–26 exhibited the same number of CRs overall, but significantly fewer double-peaked CRs compared with the rats trained on Postnatal Days 32–34. The younger rats also showed a significantly longer latency in the first peak of double-peaked CRs, indicating that the precision of timing continues to develop in parallel with the increase in the percentage of double-peaked responses (see Figure 5).

Experiment 3

The developmental differences in CR timing seen in Experiment 2 were robust but might have been specifically related to the use of a trace conditioning paradigm. Infant rats acquire long-delay and trace conditioning at approximately the same rate (Ivkovich et al., 2000), but the presence of a trace interval could impair the precision of CR timing mechanisms in the younger rats when training involves more than one ISI. The trace interval introduces a memory demand that is not present in delay conditioning paradigms (i.e., bridging the temporal gap between the CS and US). The trace procedure also has the additional cue of CS offset, which might have produced memory interference during the trace period. The younger rats may have been more sensitive to the memory demands and potential mnemonic interference that are inherent in the trace paradigm. Moreover, the presence of the tone CS in the ISI during delay conditioning could be more effective at activating pontine mossy fibers in younger rats, and thereby aid the acquisition of well-timed CRs.

Experiment 3 used a delay paradigm to examine ontogenetic changes in CR timing. The procedure was identical to that used in Experiment 2, except that the tone CS was on throughout the ISIs (see Figure 6).

Method

Subjects. Subjects were 19 pups trained on either Postnatal Days 24–26 ( $n = 9$ ) or 32–34 ( $n = 10$ ). The rats were housed in the animal

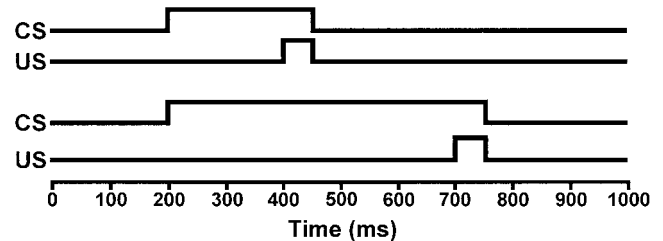


Figure 6. Diagram of the delay conditioning procedure used in Experiment 3. The time courses of the conditioned stimulus (CS) and unconditioned stimulus (US) are depicted for two different types of trials. The upper CS and US traces depict the short-CS trials, and the lower two traces depict the long-CS trials. Note that there is no time gap between the CS offset and US onset.

colony in Spence Laboratories at the University of Iowa. All rats were maintained on a 12-hr light–dark cycle (light onset at 7 a.m.) and given ad-lib access to food and water.

**Surgery and apparatus.** The surgical procedure and the apparatus were the same as in Experiments 1 and 2.

**Conditioning procedure.** The experimental design was the same as used in Experiments 1 and 2, except that the tone CS was on throughout the ISI and coterminated with the US (see Figure 6). Therefore, the durations for the tone on the two trial types were 250 and 550 ms. As in Experiments 1 and 2, every 10th trial was a CS-alone test trial. In Experiment 3, the rats were given 5 test trials with the short CS and 5 test trials with the long CS. All other aspects of the conditioning procedure were the same as used in Experiments 1 and 2.

**Results**

As in Experiment 2, the two groups of rats acquired CRs at the same rate in Experiment 3 (Figure 7). Both groups reached asymptotic levels of performance on Session 6. However, as in Experiment 2, the younger rats exhibited fewer double-peaked CRs relative to the older rats (see Figure 8). The age-related difference in the percentage of double-peaked CRs occurred during test trials with the long CS (see Figure 8). As Millenson et al. (1977) demonstrated, test trials with the short CS yielded fewer double-peaked CRs, perhaps because the offset of the short CS serves as an inhibitory stimulus. Examination of the latencies of the peaks in trials with double-peaked CRs during the last training session revealed that the younger pups generated double-peaked CRs with a first peak that followed the first onset time of the US ( $M = 209.3$  ms, 9.3 ms after US onset) and a second peak that also followed the second onset time of the US ( $M = 544.5$  ms, 44.5 ms after US onset; see Figure 9). The older rats produced CRs with a peak that preceded the first onset time of the US and a second peak that occurred at the second onset time of the US ( $M_s = 175.4$  ms, 24.6 ms before first US onset and 501.7 ms, 1.7 ms after second US onset).

As in Experiment 2, the percentage of CRs, the percentage of double-peaked CRs, and the peak latencies of the double-peaked

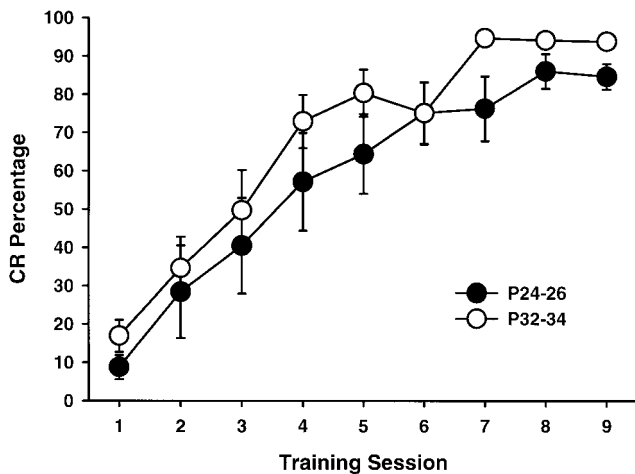
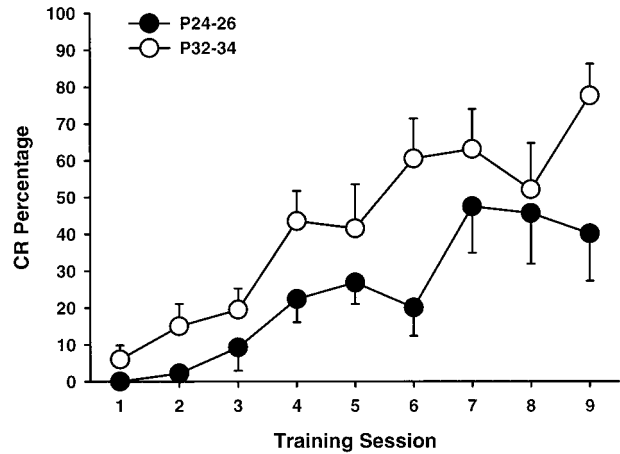


Figure 7. Mean ( $\pm$ SEM) percentage of conditioned responses (CRs) produced by rats trained on Postnatal Days (P) 24–26 or 32–34 as a function of 100-trial training sessions. The rats were given three training sessions per day (Experiment 3).

**Double Peaked CR Percentage During Long-CS Test Trials**



**Double Peaked CR Percentage During Short-CS Test Trials**

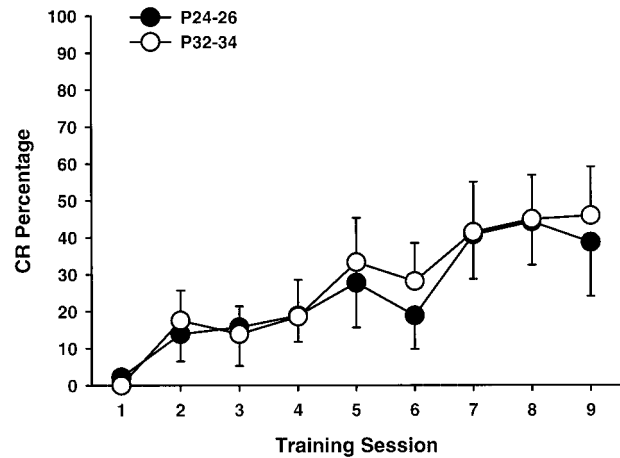


Figure 8. Mean ( $\pm$ SEM) percentage of double-peaked conditioned responses (CRs) produced by rats trained on Postnatal Days (P) 24–26 or 32–34 as a function of training sessions for trials with the long conditioned stimulus (CS; top) and short CS (bottom) in Experiment 3 (delay conditioning).

responses were examined between age groups by analysis of variance. There were no significant effects for the percentage of CRs between groups in an analysis of both trial types combined, or in separate analyses of trials with the short CS or long CS. Analysis of the percentage of double-peaked CRs on long-CS test trials revealed a main effect of age,  $F(1, 16) = 4.92, p < .05$ , which was due to a higher percentage of double-peaked CRs in the older pups. There were no age-related effects on the percentage of double-peaked CRs during test trials with the short CS. There was a significant effect of age for the analysis of peak latency for double-peaked CRs,  $F(1, 15) = 6.63, p < .03$ . This effect was due to longer latency peaks in the younger pups.

The peak latency of the single-peaked CRs on test trials with the long CS occurred between the two onset times of the US. The mean peak latency for single-peaked CRs for rats trained on

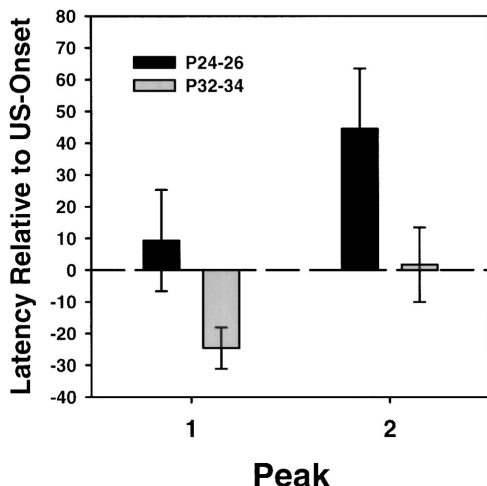


Figure 9. Mean ( $\pm$ SEM) latency of the first (left) and second (right) peak of double-peaked conditioned responses relative to the onset times of the unconditioned stimulus (US) in rats trained on Postnatal Days (P) 24–26 or 32–34 (Experiment 3, delay conditioning).

Postnatal Days 24–26 was 369 ms after CS onset, which was 169 ms after the first US onset time and 131 ms before the second US onset time. The mean peak latency for single-peaked CRs for rats trained on Postnatal Days 32–34 was 335 ms from CS onset, which was 135 ms after the first US onset time and 165 ms before the second US onset time.

Discussion

In Experiment 3, the rat pups in both age groups rapidly acquired CRs during training with a delay conditioning procedure that is similar to the procedure used by Millenson et al. (1977) in their study of conditioning with two ISIs in adult rabbits (see Figure 7). The rat pups trained on Postnatal Days 32–34 acquired CRs with two peaks at a higher rate than the rat pups trained on Postnatal Days 24–26 (see Figure 8). There was also a developmental difference in the performance of the double-peaked CRs such that the younger rats exhibited double-peaked CRs with both peaks following the onset times of the US, whereas the older pups produced CRs with a first peak that preceded the first US onset time and a second peak that occurred at the second US onset time (see Figure 9). The developmental changes in the timing precision of the peak latencies of double-peaked CRs in Experiment 3 differ from the pattern of peak latency data from Experiment 2 (cf. Figures 5 and 9). The developmental differences in the performance of double-peaked CRs between experiments are presumably related to differences between the delay and trace paradigms (see General Discussion). However, the general pattern of findings of Experiment 3 is similar to that of Experiment 2, showing that precise timing of eyeblink CRs continues to develop past the age at which conditioning with a single ISI is relatively well developed (i.e., Postnatal Day 24).

General Discussion

Eyeblink CR Timing in Adult Rats

The first experiment in this study examined eyeblink CR timing in adult rats using a slightly modified version of a temporal

uncertainty paradigm developed by Moore and Choi (1997) for use with adult rabbits. Adult rats were trained with a single 150-ms tone CS that was paired with a 50-ms periorbital shock US. The US was presented at ISIs of 200 or 500 ms. CRs with peak amplitudes that corresponded to the onset times of the US were acquired rapidly. The two peaks of the double-peaked CRs preceded the onset times of the US by 24.7 and 65.8 ms, respectively. In contrast, a previous study using adult rabbits and a similar temporal uncertainty paradigm found that the response peaks occurred at approximately the time of the onsets of the US (Moore & Choi, 1997). The discrepancy between the relative timing of the responses in the current study and that of the Moore and Choi study is primarily due to the use of different measures of the response. Moore and Choi measured movement of the eyelid, whereas the current study measured EMG activity in the eyelid muscle. It is well known that eyelid EMG precedes the onset of movement (Berthier, 1992; Pellegrini et al., 1995; Trigo et al., 1999), which accounts for much of the difference in the latencies of the CR peaks. However, there may also be species differences in response latencies.

Developmental Changes in Eyeblink CR Timing

Developmental changes in eyeblink CR timing were examined in Experiments 2 and 3. In short-delay conditioning paradigms, the eyeblink CR emerges ontogenetically between Postnatal Days 17 and 24 (Stanton et al., 1992). The rate of acquisition and level of asymptotic performance increase continuously (i.e., not as a step function) as a function of age (Freeman, Barone, & Stanton, 1995; Ivkovich et al., 2000; Stanton et al., 1998). By Postnatal Day 24, rats exhibit rapid acquisition of CRs with peaks that occur approximately 35 ms before the onset of the US (unpublished data). However, Purkinje cells in the cerebellar cortex and hippocampal neurons continue to develop past Postnatal Day 24 in rats (Anderson & Flumerfelt, 1985; Berry & Bradley, 1976; Cotman et al., 1973; Dumas & Foster, 1995; Harris & Teyler, 1984; Pokorny & Yamamoto, 1981a, 1981b). Therefore, behavioral processes that rely heavily on cerebellar cortical or hippocampal function such as CR timing might continue to develop past Postnatal Day 24. The results of Experiments 2 and 3 indicate that the precise CR timing seen as a result of training with temporal uncertainty paradigms continues to develop after Postnatal Day 24.

Rats trained on Postnatal Days 24–26 acquired CRs during both the trace version (Experiment 2) and the delay version (Experiment 3) of the temporal uncertainty paradigm at the same rate as rats trained on Postnatal Days 32–34. However, in both Experiments 2 and 3, the younger rats acquired double-peaked CRs at a significantly slower rate than the older rats. The rats trained on Postnatal Days 32–34 produced double-peaked CRs for which each peak preceded the US onset times during training with the trace procedure (Experiment 2) and the first peak preceded the first US onset time during training with the delay procedure (Experiment 3). The rats trained on Postnatal Days 24–26 exhibited a different pattern of CR peak latencies for the trace (Experiment 2) and delay (Experiment 3) training. In Experiment 2, the younger rats produced double-peaked CRs with the first peak lagging behind the first onset time of the US. In Experiment 3, the younger rats produced double-peaked CRs with both peaks lagging behind the onset times of the US. The important general finding from

Experiments 2 and 3 is that infant rats exhibited developmental changes in the acquisition and performance of double-peaked CRs. However, it is not clear why there were different developmental trends in the performance of double-peaked CRs for trace and delay paradigms.

A previous study found that the ontogenetic changes in the acquisition of eyeblink CR did not differ between trace and long-delay paradigms using a single ISI (Ivkovich et al., 2000). However, the use of two ISIs during training may have been the factor that revealed subtle differences in the ontogeny of eyeblink conditioning in trace and delay paradigms. The trace and delay paradigms used in this study differ in important ways that may account for differences in CR timing among infant rats. The trace paradigm produces a heavier burden on memory by requiring the rats to maintain a representation of the CS (or the lack of a CS) during the trace interval. The trace paradigm also presents two types of cues to the rats: the onset of the CS and its offset. A full account of the developmental differences in performance of double-peaked CRs during trace and delay conditioning paradigms will only come with further experimental analysis.

#### *Neural Basis for the Late Development of Precise CR Timing*

Experiments 2 and 3 revealed developmental changes in the percentage and performance of double-peaked CRs, but no developmental difference in the overall percentage of CRs. The developmental dissociation of overall CR production and CR timing suggests that the basic plasticity mechanisms underlying eyeblink conditioning are present by Postnatal Day 24 but continue to mature until Postnatal Day 32. The ontogenetic increase in CR timing might be related to the continued functional maturation of the cerebellar cortex and hippocampus or to the development of mechanisms underlying hippocampal interactions with the cerebellum. Developmental changes in other forebrain systems and their connections to the hippocampus or cerebellum could also influence the ontogenetic changes in CR timing.

#### *Conclusions*

The experiments in this study constitute a first step toward understanding the neural mechanisms underlying the ontogeny of eyeblink CR timing. The findings of the first experiment indicate that adult rodents acquire and perform well-timed eyeblink CRs. These findings extend the previous demonstrations of precise CR timing in rabbits and suggest that response timing could be assessed in other species such as mice. The developmental changes in acquisition and performance of CR timing indicate that there may be substantial cerebellar cortical or hippocampal functional development after Postnatal Day 24, an age at which rats demonstrate robust conditioning and precisely timed CRs when trained with a single ISI. Future studies will be directed toward further understanding the behavioral factors that influence the ontogeny of eyeblink CR timing. The additional behavioral studies will provide a framework for examining the neurobiological factors underlying the ontogeny of CR timing.

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